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Response surface optimization of the medium components for the production of biosurfactants by probiotic bacteria

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Abstract

Optimization of the medium for biosurfactants production by probiotic bacteria (*Lactococcus lactis* 53 and *Streptococcus thermophilus* A) was carried out using response surface methodology. Both biosurfactants were proved to be growth-associated, thus the desired response selected for the optimization was the biomass concentration. The selected factors based on MRS medium for *L. lactis* 53 growth were peptone, meat extract, yeast extract, lactose, ammonium citrate and KH_2PO_4. For *S. thermophilus* A based on the M17 medium, the selected factors were peptone, meat extract, yeast extract, lactose, soya peptone and sodium glycerophosphate. The optimum MRS composition was found to be 38.6 g/l peptone, 43.0 g/l lactose, 10 g/l meat extract, 5 g/l yeast extract, 1.08 g/l Tween-80®, 2 g/l KH_2PO_4, 2 g/l CH_3COONa, 2 g/l ammonium citrate, 0.2 g/l $MgSO_4{\cdot}7H_2O$ and 0.05 g/l $MnSO_4{\cdot}4H_2O$. The optimized medium allowed a mass of produced biosurfactant (milligram per gram cell dry weight) 1.6 times higher compared to MRS standard medium. The optimum medium composition for growing *S. thermophilus* A consists of 5.0 g/l peptone, 5.7 g/l lactose, 5.0 g/l meat extract, 2.5 g/l yeast extract, 5.0 g/l soya peptone, 26.4 g/l sodium glycerophosphate, 0.5 g/l ascorbic acid and 0.25 g/l $MgSO_4{\cdot}7H_2O$. With the optimization procedure a biosurfactant mass recovery 2.1 times higher was achieved. The application of response surface methodology resulted in an enhancement in biosurfactants production.

Keywords: Biosurfactant; Probiotic bacteria; Response surface methodology; Medium optimization; Experimental factorial design; Product yield

1. Introduction

Interest in biosurfactants has increased considerably in recent years, as they are potential candidates for many commercial applications in the petroleum, pharmaceuticals, biomedical and food processing industries [1]. Dairy *Streptococcus thermophilus* strains, for example, can produce biosurfactants that cause their own desorption [2]. *Lactobacillus* and *Streptococcus* species have been shown to be able to displace adhering uropathogenic *Enterococcus faecalis* from hydrophobic and hydrophilic substrata in a parallel-plate flow chamber, possibly through biosurfactant production [3]. Biosurfactants have special advantages over synthetic surfactants such as their biodegradability, lower toxicity and greater diversity as they present a much broader range of surfactant types and properties than the available synthetic surfactants [4]. Depending upon the nature of the biosurfactant and the producing microrganisms, the following patterns of biosurfactant production by fermentation are possible: (a) growth-associated production, (b) production under growth limiting conditions, (c) production by resting/nongrowing cells, and (d) production associated with the precursor augmentation. In the case of growth-associated biosurfactant production, there exists a parallel relationship between the substrate utilization, growth and biosurfactant production [5].

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Cell growth and the accumulation of metabolic products are strongly influenced by medium compositions such as carbon sources, nitrogen sources, growth factors, and inorganic salts. Thus, it is difficult to search for the major factors and to optimize them for biotechnological processes as several parameters are involved [6]. Environmental factors and growth conditions such as pH, temperature, agitation, and oxygen availability also affect biosurfactant production through their effects on cellular growth or activity [1].

The classical method of medium optimization involves changing one variable at a time, keeping the others at fixed levels. Being single dimensional, this laborious and time-consuming method often does not guarantee determination of optimal conditions. On the other hand carrying out experiments with every possible factorial combination of the test variables is impractical because of the large number of experiments required [7]. In the first screening, it is recommended to evaluate the result and estimate the main effects according to a linear model. After this evaluation, the variables that have the largest influence on the result are selected for new studies. Thus, a large number of experimental variables can be investigated without having to increase the number of experiments to the extreme [8].

The aim of the present study was to improve the standard media, using lactose as carbon source instead of glucose, for growing biosurfactant-producing lactic acid bacteria. The optimization of cellular growth of the probiotic bacteria *Lactococcus lactis* 53 and *Streptococcus thermophilus* A was achieved using a 2^{6-2} fractional factorial central composite design and surface modeling method, after establishing that their biosurfactants are growth-associated. The yields of biosurfactant production for both strains were determined before and after optimization, as well as its surface-activity. The relation between cellular growth and surface-activity of the biosurfactant in time (as a measure of its production) was determined for both strains before and after the optimization procedure.

2. Materials and methods

2.1. Strains and culture conditions

The bacterial strains *L. lactis* 53 and *S. thermophilus* A were stored at −20 °C in MRS [9] or M17 [10] broth, respectively. From frozen stock, bacteria were streaked on MRS or M17 agar plates and incubated at 37 °C. To prepare subcultures, the respective medium was inoculated with a colony from the plate and incubated overnight under the same conditions. In the experimental design assays, optimization of the standard MRS and M17 media was performed by changing the carbon source from glucose to lactose, as well as the concentrations of the key factors as described below.

2.2. Cell growth and biosurfactants production

Cellular growth was measured by optical density of the culture at 600 nm and biomass concentrations (g dry weight/l) were determined using a calibration curve. The calibration curve was calculated for each strain using dilutions of a biomass suspension with known optical density. A fixed volume of the dilutions was filtered (0.45 μm) and left to dry at 105 °C for 24 h. All the filters were weighed before the filtration and after the drying. Thus, a relationship between biomass concentration (g/l) and optical density (600 nm) can be determined.

For the bacterial strains *L. lactis* 53 and *S. thermophilus* A, 600 ml cultures in MRS and M17 broth, respectively, were grown overnight (18 h). The growth media used for the production of these biosurfactants were the standard media MRS and M17, and the optimized media obtained by experimental design for higher yields of biosurfactant production. Cells were harvested by centrifugation (10,000 × *g*, 5 min, 10 °C), washed twice in demineralized water, and resuspended in 100 ml of phosphate-buffered saline (PBS: 10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.0). The bacteria were left at room temperature for 2 h with gentle stirring for cell-bound biosurfactants release. Subsequently, the bacteria were removed by centrifugation and the remaining supernatant liquid was filtered through a 0.22 μm pore-size filter (Millipore). The supernatant was dialyzed against demineralized water at 4 °C in a Spectrapor membrane tube (molecular weight cut off 6000–8000, Spectrum Medical Industries Inc., CA) and freeze-dried.

2.3. Biosurfactants surface-activity determination

Axisymmetric drop shape analysis by profile (ADSA-P) is a technique for determining liquid surface tensions based on the shape of an axisymmetric droplet on a solid substratum. In order to measure the surface-activity of both cell-bound biosurfactants obtained in the stationary growth phase (as described previously) by ADSA-P, a 100 μl droplet of a biosurfactant solution was placed on fluoroethylene-propylene (FEP)-Teflon (Fluorplast, The Netherlands) in an enclosed chamber to prevent evaporation from the droplet. The shape of the droplet was monitored for 2 h at room temperature and the surface tension of the droplet was calculated from its shape as a function of time [11]. Surface-activity of biosurfactant produced by bacteria in time was also measured by ADSA-P. Bacterial suspensions were prepared as follows. The *L. lactis* 53 and *S. thermophilus* A were grown in 200 ml of MRS and M17 broth, respectively, inoculated with 10 ml of an overnight pre-culture. After 3, 6, 9 and 24 h, 10 ml of the culture was harvested by centrifugation (10,000 × *g*, 5 min, 10 °C) and washed twice in fresh PBS. Bacteria were counted in a Bürker-Türk counting chamber and diluted in PBS to a final concentration of 5×10^9 cell/ml,

and used immediately as described above in the ADSA-P procedure.

2.4. Mass of produced biosurfactants

In order to compare the amount of cell-bound biosurfactants produced by the bacteria grown in standard and optimized medium, the biosurfactants were released by the stationary phase cells using the PBS extraction procedure described below. Briefly, the bacteria were left at room temperature for 2 h with gentle stirring for cell-bound biosurfactants release. Subsequently, the bacteria were removed by centrifugation and the remaining supernatant liquid was filtered through a 0.22 μm pore-size filter (Millipore). The supernatant was dialyzed against demineralized water at 4 °C in a Spectrapor membrane tube (molecular weight cut off 6000–8000, Spectrum Medical Industries Inc., CA) and freeze-dried. The mass of produced biosurfactant (milligram per gram cell dry weight) was determined.

2.5. Experimental designs

Response surface methodology is a collection of mathematical and statistical techniques that are useful for the modeling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize this response [8].

2.5.1. Fractional factorial designs (FFD)

In order to identify which component(s) of the medium has a significant effect on cellular growth a first optimization step was developed. In a factorial design the influences of all experimental variables, factors, and interaction effects on the response or responses are investigated [8]. Six major components in MRS (peptone, meat extract, yeast extract, lactose, ammonium citrate and KH_2PO_4) and M17 medium (peptone, meat extract, yeast extract, lactose, soya peptone and sodium glycerophosphate) to be set as factors in the factorial designs were selected. According to factorial designs 2^6 experiments have to be performed. If the experimenter can reasonably assume that certain high-order interactions are negligible, information on the main effects and low-order interactions may be obtained by running only a fraction of the complete factorial experiment. The number of experiments can then be reduced by using only a part of the factorial designs (fractional factorial design) without loss of information about the main effects. For a moderately large number of factors, smaller fractions of the 2^k design are frequently useful [8]. Therefore, for a 2^{6-2} fractional factorial design with six factors at two levels, only 16 experimental runs are required. A first-order model was then fitted to the data obtained from the FFD experiments. Frequently, the initial estimate of the optimum operating conditions for the system will be far from the actual optimum. In such circumstances, the objective is to move rapidly to the general vicinity of the optimum. The method of steepest ascent is a procedure for moving sequentially along the path of steepest ascent, that is, in the direction of the maximum increase in the response. Further studies for the optimization involved experiments carried out along the path of steepest ascent, which means, the direction at right angles to the contour lines representing equal yield, that shows the relative amounts by which the factors have to vary in order to attain a maximum increase of responses.

2.5.2. Central composite designs (CCD)

The objective of this second experiment is to develop an empirical model of the process and to obtain a more precise estimate of the optimum operating conditions for the factors involved. This approach to process optimization is called response surface methodology and the second design is a central composite design, one of the most important experimental designs used in process optimization studies [8]. In order to describe the nature of the response surface in the optimum region, a central composite design with five coded levels was performed. For the two factors, this design was made up a full 2^2 factorial design with its four cube points, augmented with five replications of the center points and the four star points, that is, points having for one factor an axial distance to the center of $\pm\alpha$, whereas the other factor is at level 0. The axial distance α was chosen to be 1.414 to make this design rotatable. A center point is a point in which all variables are set at their mid value. Three or four center experiments should always be included in factorial designs because the risk of missing non-linear relationships in the middle of the intervals has to be minimized, and also because the repetition allows for determination of confidence intervals [8]. To estimate the optimal point, a third-order polynomial function was fitted to the experimental results.

2.5.3. Data analysis

Design-Expert 6, Trial version was used for the regression analysis of the experimental data obtained. The quality of the fit of the polynomial model equation was expressed by the coefficient of determination R^2, and its statistical significance checked by a F-test [8]. The significance of the regression coefficient was tested by a t-test. The level of significance was given as values of Prob $> F$ less than 0.1. A differential calculation was then employed for predicting the optimum point.

3. Results

3.1. Biosurfactant growth-associated production

The relation between cell growth and surface-activity of the biosurfactant in time was determined for both strains before the optimization procedure (Figs. 1A and 2A). For both strains the biosurfactant production is associated with

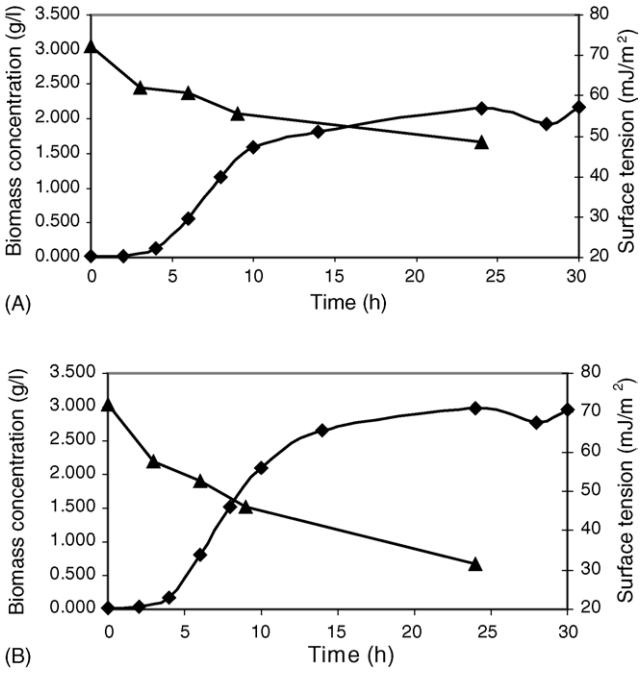

Fig. 1. Fermentation evolution for *L. lactis* 53: variation of biomass concentration (g/l) (■) and surface tension (mJ/m^2) (▲), in time. The biomass concentration is a measure of the cell growth, while surface tension is a measure of the biosurfactant activity. (A) *L. lactis* 53 grown in MRS medium before experimental design optimization of the media composition. (B) *L. lactis* 53 grown in MRS optimized by experimental design.

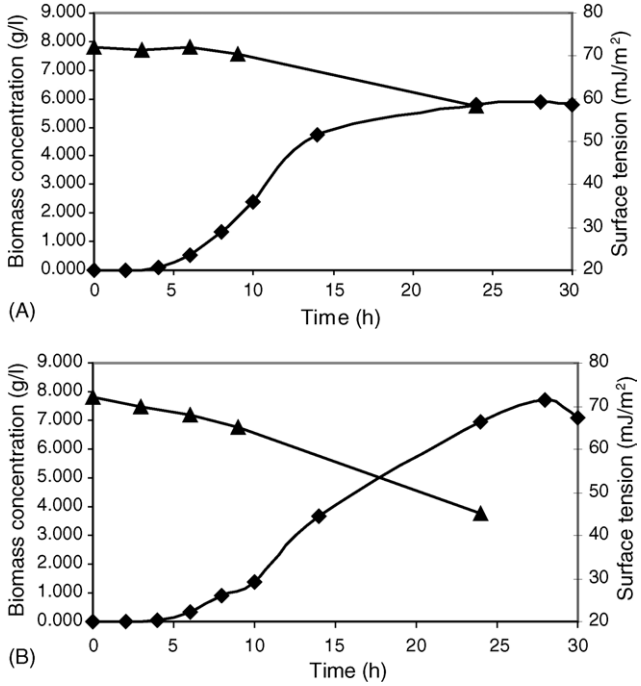

Fig. 2. Fermentation evolution for *S. thermophilus* A: variation of biomass concentration (g/l) (■) and surface tension (mJ/m^2) (▲), in time. The biomass concentration is a measure of the cell growth, while surface tension is a measure of the biosurfactant activity. (A) *S. thermophilus* A grown in M17 medium before experimental design optimization of media composition. (B) *S. thermophilus* A grown in M17 optimized by experimental design.

the cellular growth, as an increase in the biomass concentration leads to a decrease in the surface tension. In the case of a growth associated biosurfactant production there is a parallel relationship between the substrate utilization, growth and biosurfactant production [5]. The lowest values of surface tension were achieved in the stationary phase for both bacterial strains.

3.2. Effects of the different MRS or M17 medium components on cell growth

The factorial design enables the identification of the medium components that play a significant role on cell growth, as well as the ranges within the medium components vary. For each medium six components were set as variables for the optimization procedure and the concentration for each component in the medium was appropriately enlarged as the ranges for the variables. The independent variables, experimental range and levels investigated in this study, for both media, are given in Table 1. In developing the regression equation, the test variables were coded according to the equation:

$$x_i = \left(\frac{X_i - X_i^*}{\Delta X_i} \right) \quad (1)$$

where x_i is the coded value of the ith independent variable, X_i the uncoded value for the ith independent variable, X_i^* the uncoded value of the ith independent variable at the center point and ΔX_i the step change value.

Results of the experimental design performed to achieve MRS medium optimization are shown in Table 2. The biomass concentration varied markedly from 1.811 to 4.250 g/l with the different levels of components in the medium. The concentration of lactose and peptone strongly affected the cell growth, with P-values of 0.0766 and 0.0015, respectively, whereas ammonium citrate and KH_2PO_4 did

Table 1
Experimental range and levels of the independent variables (X_i and Z_i, i = 1, 2, 3, 4, 5, and 6) used in the fractional factorial design (FFD)

Independent variables (g/l)	Range and levels		
	−1	0	1
MRS medium optimization			
X_1 – peptone	5.0	10.0	15.0
X_2 – meat extract	5.0	10.0	15.0
X_3 – yeast extract	2.5	5.0	7.5
X_4 – lactose	10.0	20.0	30.0
X_5 – ammonium citrate	1.0	2.0	3.0
X_6 – KH_2PO_4	1.0	2.0	3.0
M17 medium optimisation			
Z_1 – peptone	2.5	5.0	7.5
Z_2 – meat extract	2.5	5.0	7.5
Z_3 – yeast extract	1.25	2.5	3.75
Z_4 – lactose	5.0	10.0	15.0
Z_5 – soya peptone	2.5	5.0	7.5
Z_6 – sodium glycerophosphate	9.5	19.0	28.5

Table 2
Experimental design and results of the fractional factorial design (FFD)

MRS medium optimisation

Run	x_1[a]	x_2[a]	x_3[a]	x_4[a]	x_5[a]	x_6[a]	Biomass concentration (g/l)	
							Observed[b]	Expected[c]
1	−1	−1	−1	−1	−1	−1	2.179	1.960
2	−1	+1	+1	−1	−1	−1	1.811	2.180
3	−1	+1	+1	+1	−1	+1	2.434	2.570
4	0	0	0	0	0	0	2.584	2.490
5	−1	−1	+1	−1	+1	+1	2.721	2.200
6	−1	+1	−1	+1	+1	−1	2.407	2.340
7	−1	−1	−1	+1	−1	+1	2.140	2.350
8	+1	−1	+1	+1	−1	−1	4.250	3.480
9	0	0	0	0	0	0	2.491	2.490
10	+1	+1	+1	−1	+1	−1	2.963	3.060
11	0	0	0	0	0	0	2.407	2.490
12	+1	−1	+1	−1	−1	+1	2.800	3.060
13	+1	+1	−1	+1	−1	−1	3.393	3.200
14	−1	−1	+1	+1	+1	−1	2.096	2.620
15	+1	+1	+1	+1	+1	+1	3.565	3.450
16	+1	−1	−1	+1	+1	+1	2.968	3.230
17	+1	+1	−1	−1	−1	+1	2.582	2.780
18	−1	+1	−1	−1	+1	+1	2.366	1.930
19	+1	−1	−1	−1	+1	−1	2.588	2.840

M17 medium optimisation

Run	z_1[a]	z_2[a]	z_3[a]	z_4[a]	z_5[a]	z_6[a]	Biomass concentration (g/l)	
							Observed[b]	Expected[c]
1	−1	+1	−1	−1	+1	+1	5.558	5.720
2	−1	−1	+1	−1	+1	+1	5.100	5.880
3	0	0	0	0	0	0	4.627	4.480
4	0	0	0	0	0	0	6.489	4.480
5	−1	+1	−1	+1	+1	−1	0.840	−0.053
6	−1	−1	−1	+1	−1	+1	4.912	3.680
7	0	0	0	0	0	0	3.866	4.480
8	+1	−1	+1	+1	−1	−1	1.056	1.480
9	−1	−1	+1	+1	+1	−1	1.045	0.100
10	+1	+1	−1	+1	−1	−1	0.798	1.320
11	+1	+1	+1	−1	+1	−1	3.471	3.530
12	+1	+1	−1	−1	−1	+1	7.782	7.100
13	+1	−1	+1	−1	−1	+1	8.405	7.250
14	0	0	0	0	0	0	2.945	4.480
15	−1	+1	+1	+1	−1	+1	3.068	3.840
16	−1	−1	−1	−1	−1	−1	2.489	3.320
17	+1	+1	+1	+1	+1	+1	4.334	3.880
18	+1	−1	−1	−1	+1	−1	3.889	3.370
19	+1	−1	−1	+1	+1	+1	1.901	3.720
20	−1	+1	+1	−1	−1	−1	2.960	3.480

[a] The coded variables x_i and z_i ($i = 1, 2, 3, 4, 5$, and 6) are defined in Table 1.
[b] Observed biomass concentration stands for the experimental data.
[c] Expected biomass concentration is calculated from the first-order model approach (Eqs. (2) and (3)).

not significantly influence cell growth. Furthermore, it was found that the yeast extract is more important for the cell growth than the meat extract. The values of the regression coefficients were calculated and the response variable Yb^*_{Ll} could be written as a fit of the experimental data:

$$Yb^*_{Ll} = 2.70 + 0.43x_1 - 0.01x_2 + 0.13x_3 + 0.20x_4 + 0.01x_5 - 0.01x_6 \quad (2)$$

The results for M17 medium optimization demonstrated that the biomass concentration varied markedly from 0.798 to 8.405 g/l with the different levels of components in the medium. The concentration of lactose and sodium glycerophosphate strongly affected the cell growth, with P-values of 0.0009 and 0.0003, respectively. All the other medium components did not significantly influence cell growth. The desired response variable (Yb^*_{St}) was set as biomass concentration (g/l) in the stationary phase. The values of the regression coefficients were calculated and the response variable Yb^*_{St} could be written as a fit of the experimental data:

$$Yb^*_{St} = 3.60 + 0.35z_1 + 0.01z_2 + 0.08z_3 - 1.361z_4 - 0.33z_5 + 1.531z_6 \quad (3)$$

For the MRS medium optimization the regression analysis of the FFD showed that peptone (x_1) and lactose (x_4) were significant at the probability levels of 99 and 95%, respectively, for cell growth and proved to be the two most important components of the medium. All the other components of the medium, except for the yeast extract, were not found to be significant at the probability level of 90% for cell growth. Table 3 presents the coefficient of determination R^2 of the model to be 0.68, which means that the model explains 68% of the variability in the data. This ensured a satisfactory adjustment of the first order model to the experimental data. The statistical significance of the model equation was also confirmed by an F-test. The model F-value of 3.81 implies the model is significant, which means that there is only 2.64% chance that a model F-value this large could occur due to noise. The lack of fit F-value of 26.62 is significant, which means that there is only a 3.67% chance that this value could occur due to noise. A significant lack of fit is bad because we want the model to fit. The purpose of statistical analysis is to determine which experimental factors generate signals, which are large in comparison to the noise. The adequate precision value measures signal to noise ratio and a ratio greater than 4 is desirable. The adequate precision value shows an adequate signal, which means this model can be used to navigate the design space and further optimization. Fig. 3(A) represents the relationship between the observed biomass concentration values and the expected values determined by the model

Table 3
Analysis of variance (ANOVA) for the first order models determined from the fractional factorial design (FFD)

Values	MRS medium optimisation	M17 medium optimization
R^2	0.68	0.80
Adjusted R^2	0.50	0.69
Predicted R^2	−0.01	0.47
Adequate precision	5.79	9.38
Model F-value	3.81	7.78
Lack of fit F-value	26.62	0.56

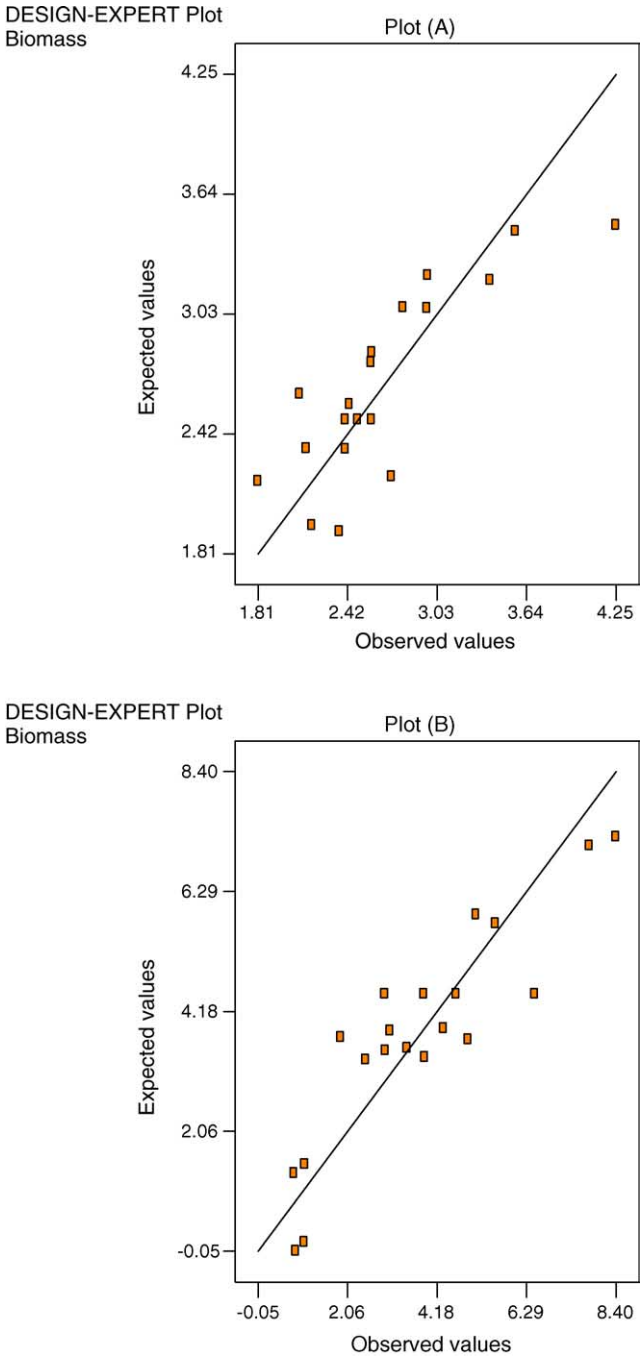

Fig. 3. Plots of Observed vs. expected biomass concentration (g/l) values for *L. lactis* 53 (A) and *S. thermophilus* A (B). The biomass concentration is the response variable of interest. The expected biomass concentration values are determined by the model equations determined for fractional factorial design (FFD).

Eq. (2) for *L. lactis* 53. It can be observed that most points are nearby the line adjustment which means that the values determined experimentally are similar to those determined by the model. Also for *S. thermopilus* A it can be observed in Fig. 3B the same tendency.

For the M17 medium optimization the regression analysis of the *FFD* showed that lactose (z_4) and sodium glycerophosphate (z_6) were significant at the probability level of 99% for cell growth and consequently the two most important components. All the other components of the medium were not found to be significant at the probability level of 90% for cell growth. The ANOVA summarized in Table 3 showed a coefficient of determination R^2 of 0.80, which means that the model explains 80% of the variability in the data. The model F-value of 7.78 implies that the model is significant and there is only 0.14% chance that this value could occur due to noise. The lack of fit F-value of 0.56 is not significant relative to true pure error, and there is a 77.9% chance that this value could occur due to noise. This model was found to be adequate to navigate the design space and further optimization.

3.3. The path of steepest ascent

The path of steepest ascent was determined by first-order model (Eqs. (2) and (3)) and regression analysis for both bacterial strains. Besides the previously determined significant factors (peptone (x_1) and lactose (x_4) for MRS medium optimization; lactose (z_4) and sodium glycerophosphate (z_6) for M17 medium optimization), all the other components were fixed at the center level of the FFD because they were not significant at the probability level of 90% for cell growth. According to the signs of their main effects, the concentrations of the significant factors were increased or decreased, in order to achieve a positive consequence in the response variable. In MRS medium optimization, peptone and lactose were increased serially by 0.5 and 0.25%, respectively, while for M17 medium optimization lactose was decreased serially by 2.0%, and sodium glycerophosphate was increased serially by 1.0%. The higher biomass concentration (2.453 g/l) has been reached with 30 g/l peptone and 38.6 g/l lactose for MRS medium optimization. For M17 medium optimization, 3.2 g/l lactose and 26.6 g/l sodium glycerophosphate allowed a 6.656 g/l biomass concentration.

3.4. Central composite design (CCD)

By determining the path of steepest ascent the vicinity of the optimum was reached. Thus, for MRS medium optimization, the levels of the two significant variables, peptone (x_1) and lactose (x_4) were further optimized using a central composite design. The ranges of the variables are 30–40 g/l for peptone, and 34–43 g/l for lactose. The experimental design and the results are presented in Table 4. The experimental results of the CCD were fitted with a third-order polynomial function for estimation of biomass concentration:

$$Yb_{Ll} = 2.55 + 0.46x_1 + 0.15x_4 + 0.15x_1x_4 - 0.15x_1^2 + 0.11x_4^2 - 0.26x_1^3 - 0.11x_4^3 \quad (4)$$

The model adequacy was checked and it was found to be adequate, the goodness of fit of the model was expressed by

Table 4
Experimental design and results of the central composite design (CCD)

MRS medium optimization

Run	x_1[a]	x_4[a]	Biomass concentration (g/l)	
			Observed[b]	Expected[c]
1	0	0	2.463	2.550
2	1.414	0	2.069	2.170
3	0	1.414	2.564	2.670
4	+1	+1	3.016	2.910
5	−1	+1	2.305	2.200
6	0	0	2.469	2.550
7	+1	−1	2.630	2.520
8	0	−1.414	2.778	2.880
9	−1.414	0	2.232	2.340
10	0	0	2.832	2.550
11	0	0	2.499	2.550
12	−1	−1	2.536	2.430
13	0	0	2.501	2.550

M17 medium optimisation

Run	z_4[a]	z_6[a]	Biomass concentration (g/l)	
			Observed[b]	Expected[c]
1	0	0	6.138	6.10
2	0	0	5.960	6.10
3	1.414	0	5.862	5.64
4	+1	+1	6.201	6.42
5	0	−1.414	6.290	6.07
6	0	0	6.184	6.10
7	0	0	6.132	6.10
8	0	1.414	6.042	5.82
9	−1	+1	5.711	5.93
10	0	0	6.100	6.10
11	−1	−1	5.613	5.84
12	+1	−1	5.305	5.53
13	−1.414	0	6.422	6.20

[a] The coded variables x_i ($i = 1$ or $i = 4$) and z_j ($j = 4$ or $j = 6$) are defined in Table 1.
[b] Observed biomass concentration stands for the experimental data.
[c] Expected biomass concentration is calculated from the third-order model approach (Eqs. (4) and (5)).

the coefficient of determination R^2, which was calculated to be 0.75, indicating that 75% of the variability in the response could be explained by the model. The P-value obtained for the significant variables was 0.0870. This proves that the model equation as expressed in Eq. (4) provides a suitable model to describe the response of the experiment pertaining to cell growth. Fig. 4A shows the surface response plot of the model equation. From equations derived by differentiation of Eq. (4), we can obtain the maximum point of the model, which was 38.6 g/l of peptone and 43.0 g/l lactose. The model predicted a maximum response for biomass concentration of 2.9722 g/l for this point. In order to confirm the predicted results of the model, experiments using the medium representing this maximum point were performed and a value of 3.213 g/l (triplicate experiments were carried out and correspond within 15%) was obtained. Thus, the optimum medium composition for growing *L. lactis* 53 consists of: 38.6 g/l peptone, 43.0 g/l lactose, 10 g/l meat extract, 5 g/l yeast extract, 1.08 g/l Tween-80®, 2 g/l KH_2PO_4, 2 g/l

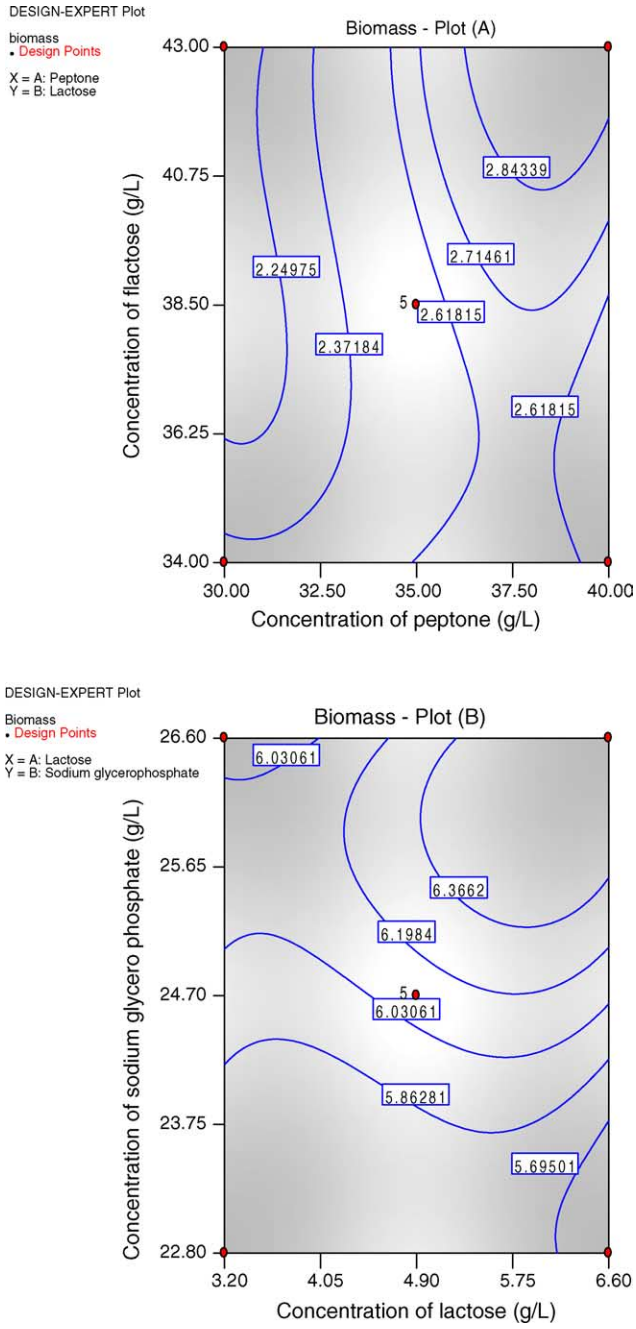

Fig. 4. Response surface contour plots of biomass concentration (g/l) for *L. lactis* 53 and *S. thermophilus* A. The biomass concentration is the response variable of interest. The contour plots represent the effect of the significant variables and their interaction in the response variable. All the other variables non significant are held at zero level of the central composite design (CCD). (A) The effect of peptone, lactose and their mutual interaction on biomass concentration for *L. lactis* 53. (B) The effect of lactose, sodium glycerophosphate and their mutual interaction on biomass concentration for *S. thermophilus* A.

CH_3COONa, 2 g/l ammonium citrate, 0.2 g/l $MgSO_4{\cdot}7H_2O$ and 0.05 g/l $MnSO_4{\cdot}4H_2O$.

For the M17 medium optimization the procedure adopted was similar to the one described above, thus the levels of the two significant variables, lactose (z_4) and sodium

glycerophosphate (z_6) were further optimized using a central composite design. The ranges of the variables are 3.2–6.6 g/l for lactose, and 22.8–26.6 g/l for sodium glycerophosphate. The experimental design and the results are also presented in Table 4. Also for the M17 medium optimization a third-order polynomial function was fitted to the CCD data for estimation of biomass concentration:

$$Yb_{St} = 6.10 + 0.29z_4 + 0.58z_6 + 0.20z_4z_6 - 0.09z_4^2 - 0.08z_6^2 - 0.24z_4^3 - 0.34z_6^3 \quad (5)$$

The model adequacy was checked and it was found to be adequate, the goodness of the fit was expressed by the coefficient of determination R^2, which was 0.62, indicating a 62% of variability in the response was explained by the model. Fig. 4B shows the surface response plot of the model equation. The *P*-value obtained for the significant variables was 0.1338. Differentiation of Eq. (5) allowed the determination of the maximum point of the model, which was 5.7 g/l of lactose and 26.4 g/l sodium glycerophosphate. The model predicted a maximum response for biomass concentration of 6.4983 g/l for this point. The validation of the model was performed using the medium representing this maximum point and a value of 6.184 g/l (triplicate experiments were carried out and correspond within 15%) was obtained. Thus, the optimum medium composition for growing *S. thermophilus* A consists of: 5.0 g/l peptone, 5.7 g/l lactose, 5.0 g/l meat extract, 2.5 g/l yeast extract, 5.0 g/l soya peptone, 26.4 g/l sodium glycerophosphate, 0.5 g/l ascorbic acid and 0.25 g/l $MgSO_4{\cdot}7H_2O$.

Fig. 5 represents the relationship between the observed biomass concentration values and the expected values determined by the model Eqs. (4) and (5) for *L. lactis* 53 and *S. thermophilus* A, respectively. It can be observed that most points are nearby the line adjustment which means that the values determined experimentally are similar to those determined by the model.

3.5. Biosurfactants mass recovery and surface-activity

After the optimization procedure the evaluation of fermentation for both probiotic strains was performed (Figs. 1B and 2B). Comparing results before and after the optimization procedure for *L. lactis* 53 (Fig. 1A and B.), it can be observed for the same fermentation time, a higher biomass concentration and surface-activity of the biosurfactant. The optimization procedure allowed an increase of 1.6 times in the mass recovery of biosurfactant produced (milligram per gram cell dry weight). Also for *S. thermophilus* A (Fig. 2A and B), with the optimization procedure a higher biomass concentration and surface-activity of the biosurfactant was achieved. The mass of biosurfactant produced (milligram per gram cell dry weight) increased 2.1 times. For both bacterial strains a stronger decrease in the surface tension along the fermentation before the optimization procedure was observed.

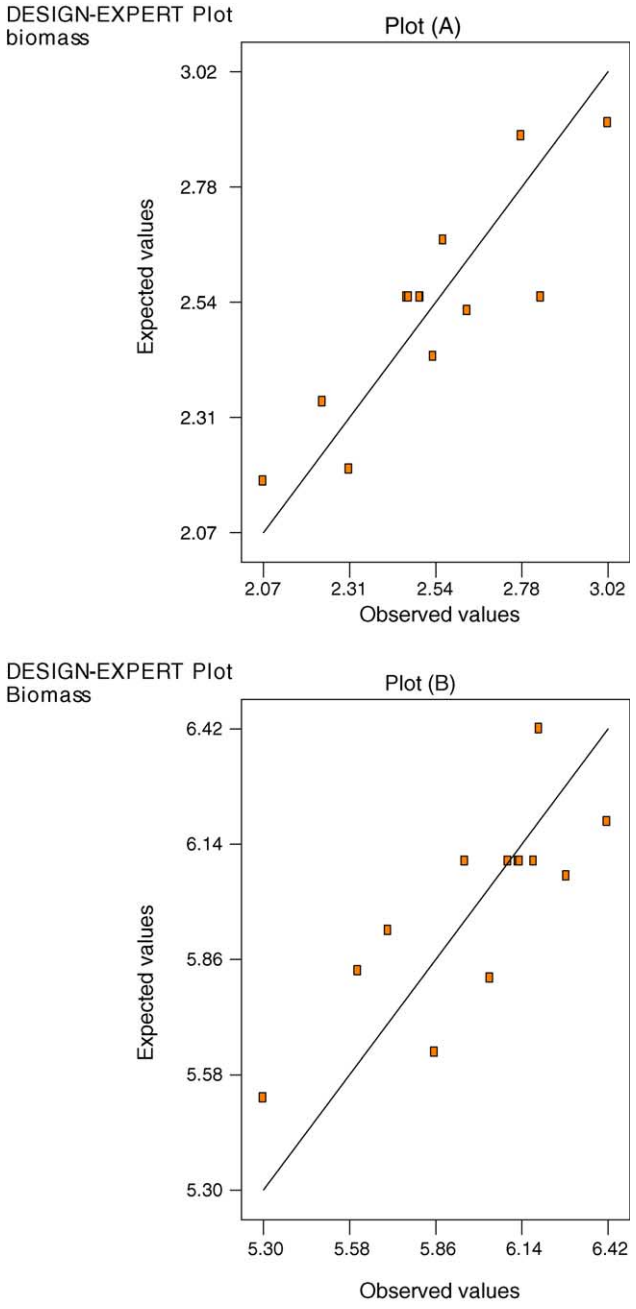

Fig. 5. Plots of observed vs. expected biomass concentration (g/l) values for *L. lactis* 53 (A) and *S. thermophilus* A (B). The biomass concentration is the response variable of interest. The expected biomass concentration values are determined by the model equations determined for central composite design (CCD).

4. Discussion

Biosurfactants produced by the probiotic bacteria *L. lactis* 53 and *S. thermophilus* A were found to be growth-associated, the biosurfactant yield of production was increased using a response surface optimization of medium composition for cell growth. Growth-associated biosurfactant production has been described for the release of biodispersan by *Acinetobacter calcoaceticus* [5]. In

addition, biosurfactant production may occur, or be stimulated, by growing the microbial cells under growth-limiting conditions. *Pseudomonas aeruginosa* shows an overproduction of rhamnolipid when the culture reaches the stationary growth phase due to limitation of the nitrogen source [5]. In our study, a direct relation exists between biosurfactant production (shown by a decrease in the surface tension) and cell growth during the fermentation process, thus the biosurfactants are growth-associated. Growth-associated biosurfactant production has been described for the production of biodispersan by *Acinetobacter calcoaceticus* [5]. In addition, biosurfactant production may occur, or be stimulated, by growing the microbial cells under growth-limiting conditions. *Pseudomonas aeruginosa* shows an overproduction of rhamnolipid when the culture reaches the stationary growth phase due to limitation of the nitrogen source [5]. Velraeds et al. [3] showed that biosurfactant release by lactobacilli is maximum for cell in the stationary phase, thus a growth-associated biosurfactant production. Hence, our present observation that cell-bound biosurfactant production by *L. lactis* 53 and *S. thermophilus* A is maximal for stationary phase cells is in accordance with the general notion on this point in the literature. Moreover, a direct relation exists between biosurfactant production (shown by a decrease in the surface tension) and cell growth along the fermentation process, thus the biosurfactants are growth-associated.

In this study we focused on the optimization of the medium compositions for cell growth, although process parameters also play an important role and could as well be improved. Optimization through factorial design and response surface analysis is a common practice in biotechnology and various research workers have applied this technique for the optimization of culture conditions [6,7,12,13], such as pH, temperature, aeration [14] and feeding rates [15]. The approach used in this study allowed the determination of the medium compositions that give the highest biomass concentration for *L. lactis* 53 and *S. thermophilus* A. In both cases, suitable models were found to describe the response of the experiments pertaining to cell growth, as the values obtained experimentally are in accordance with the expected values determined by the models. The models were validated by comparing the observed and predicted values in the optimum point, and a deviation of about 5% was found. The optimization procedure allowed an increase in biomass concentration and surface-activity of the biosurfactant.

The low level of biosurfactants produced have greatly hampered research on the role of biosurfactants; however, a number of attempts have been made to increase biosurfactant productivity by manipulating physiological conditions and medium composition. Recent developments in the area of optimization of fermentation conditions have resulted in a significant increase in production yields, making them more commercially attractive. These developments include for example, the use of a fed batch technique in which the yield of sophorolipids by *T. bombicola* increased from 0.37 g per gram substrate in batch culture to 0.6 g per gram substrate [16]. In the present study it was achieved for both bacterial strains an increase about 2 times in the mass of produced cell-bound biosurfactant (milligram) per gram cell dry weight. It is not surprising the increase in the cell-bound biosurfactant mass recovery with the optimization procedure, as it is a growth-associated biosurfactant production and the cell growth was improved. However, it is interesting to notice that the change in the carbon source (from glucose to lactose) induced the cells to produce more biosurfactant. Lactic acid bacteria ferment sugars via different pathways and are also capable of forming other products, e.g. flavors such as diacetyl and acetoin, bacteriocins or biosurfactants. The different carbon sources give varying amounts of by-products [17]. Hence, it can be speculated that the use of lactose as carbon source instead of glucose induced the cells to use another metabolic pathway, and therefore the amount of mass of cell-bound biosurfactant produced milligram per gram cell dry weight varied. Lactic acid bacteria have already proven to be ideal hosts for metabolic engineering. The efficacy of metabolic engineering of lactic acid bacteria for the increased production of biosynthetic metabolites is yet to be demonstrated, but based on the results gathered in this study it seems to be an interesting approach for developing new strategies of biosurfactant production. Moreover, since both bacterial strains shown higher amounts of cell-bound biosurfactant produced with the optimized medium, this study constitutes a step in developing strategies to produce biosurfactants from cheese whey by *L. lactis* 53 and *S. thermophilus* A. Whey is a waste product from cheese production normally used as animal feed, which contains proteins, salts and lactose. Sophorolipids production using whey was reported by Otto and his co-workers [18].

In conclusion, using the method of experimental factorial design and response surface analysis, it was possible to determine optimal operating conditions to obtain a higher cellular growth, thus a higher cell-bound biosurfactant production yield. The validity of the model was proven by fitting the values of the variables in the model equation and by actually carrying out the experiment at those values of the variables.

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